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12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

To elucidate the molecular mechanisms by which HER2/HRG influence migratory potential of breast cancer cells, we have used phospho-specific antibodies against c-Src and FAK kinases. HER2, HRG system differentially regulate signaling from FAK by selectively dephosphorylating or activating some tyrosine residues and thus increase their migratory potential rather than adhesion. HRG promoted association of Tyr phosphatase PTP1D with HER2 and PTP1D has a role in HRG mediated dephosphorylation of FAK. HER2/HRG signaling selectively upregulated Tyr phosphorylation of c-Src at Tyr-215 located within the SH2 domain, increased c-Src kinase activity and selectively upregulated Tyr phosphorylation of FAK at Tyr-861. These findings suggest that HER2/HRG influence metastasis of breast cancer cells through a novel signaling pathway involving phosphorylation of FAK tyrosine 861 via activation of c-Src tyrosine 215. Phosphospecific antibodies against the FAK and c-Src signaling molecules may potentially be used as an effective reagents to screen/identifying the putative metastatic/motile potential of breast tumors. (The U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0646 supported this work.)

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Title: A molecular approach for metastatic progression of breast cancer

PI: Ratna K Vadlamudi, Ph.D.

Report Period: June 01, 2001 to May 31, 2002 (1st Year)

INTRODUCTION:

Human Epidermal growth factor Receptor-2 (HER2) and Heregulin (HRG) are implicated in the increased progression and metastasis of human breast tumors. Focal adhesion kinase (FAK), a non-receptor tyrosine kinase present in focal adhesion complex, is implicated in the regulation of cell motility, adhesion, and anti-apoptotic signaling and is overexpressed in a number of human breast tumors. This proposal is aimed to develop a model to delineate the molecular mechanisms by which HER2, HRG utilize FAK to promote metastasis. ***My hypothesis was that HER2, HRG system differentially regulate signaling from FAK by selectively dephosphorylating some tyrosine residues and thus increase their metastatic potential rather than adhesion.***

Two model systems were used to study the role of FAK in breast cancer progression.

a) Non-invasive MCF-7 cells and HRG treatment, b) NIH 3T3 cells which express vector or activated HER2/neu.

The scope of this proposal was to undertake the following five tasks:

Task 1. Analyze site specific tyrosine phosphorylation of FAK by using phosphospecific antibodies

Task 2. Analyze the status of tyrosine phosphorylation of known FAK substrates

Task 3. Analyze activation of down stream pathways known to be activated.

Task 4. Analyze the localization of FAK and c-Src using confocal microscopy in both models.

Task 5. Analyze HER2, HRG regulation of known tyrosine phosphatases

BODY:

Task 1. To understand the molecular regulation of FAK by HER2/HRG system we have initially utilized MCF-7 model system. Using a series of commercially available phosphospecific antibodies raised against distinct sites of FAK, we analyzed the status of phosphorylation of FAK by western blotting. Our results demonstrate that HRG differentially regulates the site-specific phosphorylation of the focal adhesion components focal adhesion kinase (FAK) in a dose-dependent manner. HRG at sub-optimal doses (0.01 and 0.1 nM) induced phosphorylation of FAK at Tyr-577, Tyr-925, Tyr-407 and induced formation of well-defined focal points in breast cancer cell line MCF-7. HRG at a dose of 1 nM, which increases migratory potential of breast cancer cells increase phosphorylation of FAK at Tyr-861 but it selectively dephosphorylated FAK at Tyr-577, Tyr-407, Tyr-925. Activation of FAK at Tyr-397 remained unaffected by HRG stimulation.

Task 2. To study the effect of changes of FAK tyrosine phosphorylation on its substrate phosphorylation, we have examined the status of tyrosine phosphorylation of FAK substrates paxillin and Src using tyrosine site specific antibodies. Similar to its effect on FAK, 0.01 nM HRG stimulated Tyr-31 phosphorylation of paxillin, but 1 nM HRG reduced the level of Tyr-31 phosphorylation. Further HRG selectively enhanced Src Tyr-215 tyrosine phosphorylation only at the high dose. At 0.01 nM HRG, FAK and paxillin were transiently tyrosine phosphorylated at FAK Tyr-577, FAK Tyr-407 and paxillin Tyr-31, but no activation of Src phosphorylation was seen. Despite the activation of Src 215 phosphorylation by 1 nM HRG, no increase in the phosphorylation of its substrates FAK or paxillin was observed except increase in the tyrosine phosphorylation of FAK at Tyr 861.

Task 3. We examined the temporal relationship between FAK and paxillin tyrosine phosphorylation and the signaling pathways activated by HRG. HRG enhanced the phosphorylation of p42^{MAPK} and Akt (as a marker of PI-3 kinase activation) in a dose-dependent manner, with highest activation at 1 nM HRG, however p42^{MAPK} was only transiently

activated at 0.01 nM HRG. p38^{MAPK} was only activated at 1 nM HRG. To explore the possibility that differences in the Fak phosphorylation is due to different dimer formation of HER receptors, MCF-7 cells were treated with different doses of HRG, four HER members were immunoprecipitated using specific mAbs, and the tyrosine phosphorylation of each receptor was analyzed by blotting with anti-tyrosine mAb (Fig. 4B). The optimal dose of HRG predominantly increased the phosphorylation of HER2 and HER3, and 0.01 and 0.1 nM HRG significantly increased the tyrosine phosphorylation of HER1 and HER2. These results suggested that at a suboptimal HRG dose, signaling events were generated via EGFR/HER2 complexes. At a optimal dose signaling events may have been generated primarily by the formation of HER2/HER3 complexes and possibly from HER4/HER2 heterodimers, which may play a role in tyrosine phosphorylation of FAK and paxillin.

Task 4: To analyze the significance of HRG-mediated changes in the tyrosine phosphorylation of FAK and paxillin, *in vivo*, we examined the existence of these events. In control cells, immunostaining of FAK Tyr-577 and Tyr-925 and paxillin Tyr-31 was predominantly colocalized with focal adhesion complex dots however, low dose of HRG (0.01 nM) increased staining for all three sites while optimal dose (1nM) caused a dramatic loss of staining intensity. Analysis of the morphology of the focal contacts revealed that at suboptimal doses (0.01, 0.1 nM), HRG-activated cells were anchored to the substratum by mature focal adhesion points, represented by long, stripe-like shapes at the periphery of each unpolarized cell. In contrast, when the cells are activated with optimal doses of HRG (1 nM), small focal adhesion points accumulated at one pole of the cell, corresponding to its leading edge. These points represent very dynamic, immature focal adhesion sites reminiscent of a motile cell phenotype with distinct changes in cell shape.

Task 5: To examine the possibility that HRG at optimal dose (1nM) activates a phosphatase which in turn dephosphorylates FAK, we analyzed tyrosine phosphatase activity in HRG treated cells. HRG increased the phosphatase activity in a dose dependent manner. HRG at concentrations which promoted migration of breast cancer cells, induced activation of and increased association of tyrosine phosphatase PTP1D with HER2 but decreased association of HER2 with FAK. Expression of dominant-negative PTP1D blocked HRG-mediated dephosphorylation of FAK and paxillin, leading to persistent accumulation of mature focal points.

Report Period: June 01, 2002 to May 31, 2003 (Year 2, unfunded extension)

The scope of the second year of this unfunded extension was to undertake the following tasks:

Task 6. Validate the observed finding of differential FAK tyrosine phosphorylation in the HRG model system in a second model system using NIH 3T3 cells which express vector or activated HER2/neu.

Task 7. Examine the role of Src Tyr-215 phosphorylation on Src kinase activity by performing invitro kinase assays in the presence and absence of Src kinase inhibitor PP1.

Task 8. Explore the mechanism by which HRG/HER2 system upregulate tyrosine phosphorylation of FAK Tyr-861

Task 6. To examine whether the changes seen in the tyrosine phosphorylation of FAK, Src with HRG treatment in MCF7 cells also occurs with HER2 activation, we analyzed the effect of HER2 overexpression on the status of Src Tyr-215 and FAK Tyr-861 using a well-characterized NIH3T3 cell line, B104 that expresses activated HER2. B104 cells exhibited elevated phosphorylation of c-SrcTyr-215 and FAK Tyr-861 compared to the phosphorylation in control NIH 3T3 cells. Minimal or no change in the status of tyrosine of FAK at Tyr-577, Tyr-925 and Tyr-397 was observed. To confirm the presence of and determine the subcellular localization of FAK Tyr-861 phosphorylation in

NIH3T3 and B104 cells, we performed immunofluorescence studies followed by confocal microscopy. Results showed increased FAK Tyr-861 phosphorylation in B104 cells and was primarily localized in the cytoplasm as compared to a lower amount of the phosphorylated FAK Tyr-861 distributed in a distinct dot-like pattern in the parental NIH-3T3 cells. These findings suggested that active HER2 signaling mimics the events of HRG-signaling including phosphorylation of c-Src Tyr-215 and FAK Tyr-861.

Task 7. To examine the possible participation of Src kinase in the HER2/HRG signaling, we examined whether the increased c-Src Tyr-215 phosphorylation observed with HRG signaling correlates with Src kinase activity as assessed by in vitro kinase assay. Results show that HRG increased src kinase activity in a dose dependent manner and the activity correlated well with the increase in Src Tyr-215. Confocal microscopy analysis of HRG-treated MCF-7 cells also demonstrated elevated c-Src Tyr-215 phosphorylation. HRG treatment significantly induced the formation of motile structures such as ruffles, and importantly, a significant pool of activated c-Src Tyr-215 was localized in these structures. HRG increased FAK Tyr-861 phosphorylation in time dependent manner with maximal activation at 15 min and HRG mediated increase in FAK Tyr-861 was blocked by pretreatment of cells with Src inhibitor PP2. These results suggested that HRG may activate c-Src kinase via phosphorylation of Tyr-215 and that functionally activated c-Src kinase localizes to ruffles and may have a role in the FAK Tyr phosphorylation at Tyr-861.

Task 8. To mechanistically link HRG mediated induction of phosphorylation at Src Tyr-215 to the FAK Tyr-861 phosphorylation, we have generated a c-Src construct in which tyrosine of Src at 215 was mutated to phenylalanine (Y215F). Using this mutant we have analyzed the HRG mediated signaling to FAK at Tyr 861. Results suggest that expression of wild type c-Src substantially increased both basal and HRG mediated phosphorylation of FAK at Tyr-861 while expression of Src Tyr 215 Phe mutant failed to increase HRG mediated up regulation of phosphorylation of FAK at Tyr-861. Confocal microscopy analysis also showed that HRG induced phosphorylation of FAK Tyr-861 as compared to control. Furthermore, HRG-induced ruffle formation as well as FAK Tyr-861 phosphorylation was blocked by pretreatment of cells with the Src-specific inhibitor PP2. Together these results suggested that HRG-induced stimulation of FAK Tyr-861 phosphorylation may be mediated by c-Src kinase via its activation by phosphorylation at Tyr-215.

LIST OF PERSONEL:

Neeta Sharma, SS# 093 72 8379, Sr. Research Assistant, 100% effort,

KEY RESEARCH ACCOMPLISHMENTS:

- HRG induces a biphasic response on specific Tyr phosphorylation sites of FAK and paxillin.
- HRG mediated dephosphorylation of FAK involves PTP1D signaling
- HRG activates c-Src kinase via phosphorylation of Src Tyr-215
- HRG mediated ruffle formation in part is dependent on Src Tyr-215
- HRG/HER2 signaling utilize Src Tyr-215 pathway to selectively modulate FAK Tyr-861 phosphorylation

REPORTABLE OUTCOMES::

This study resulted in the following publications:

Vadlamudi RK, Adam I, Nguyen D, Wang R, Santos M, and Kumar R. 2002. Differential regulation of components of the focal adhesion complex by Heregulin: Role of phosphatase SHP-2. *J. Cell. Physiol.* 9:1-11.

Vadlamudi RK, Sahin AA, Adam I, Nguyen D, Wang RA, and Kumar R. 2003. Heregulin and HER2 signaling selectively activates c-Src phosphorylation at Tyrosine 215. FEBS Letters 543, 76-80.

CONCLUSIONS

Our findings suggest that HER2/HRG systems differentially regulate signaling from focal adhesion complexes through selective phosphorylation or dephosphorylation FAK, its substrate paxillin and its kinase Src. HRG at 0.01 nM promoted formation of mature focal points and increased adhesion of cells to the substratum. HRG at 1 nM concentration showed motile phenotype, decreased adhesion to the substratum. Mechanistic studies revealed that HRG and HER2 signaling uniquely increased Tyr phosphorylation of c-Src at Tyr-215 located within the SH2 domain, increases c-Src kinase activity and selectively upregulates Tyr phosphorylation of FAK at Tyr-861. HRG signaling also promotes dephosphorylation of specific Tyr residues on FAK via activation of tyrosine phosphatase PTP1D. These findings suggest that HER2/HRG influence metastasis of breast cancer cells through a novel signaling pathway involving selective phosphorylation/dephosphorylation of FAK via activation of c-Src kinase and phosphatase PTP1D. The results from this study also suggest that FAK Tyr-861 and Src Tyr-215 as potential new targets for therapeutic intervention and also to use as a prognostic marker of metastatic potential of HER2, HRG driven tumors.

APPENDICES:

1. A copy of the article published in Journal of Cellular Physiology
2. A copy of the article published in FEBS letters
3. A copy of the abstract presented in Era of Hope Breast Cancer Meetings

Heregulin and HER2 signaling selectively activates c-Src phosphorylation at tyrosine 215

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Abstract To elucidate the molecular mechanisms by which human epidermal growth factor receptor/hergulin (HER2/HRG) influence the migratory potential of breast cancer cells, we have used phospho-specific antibodies against c-Src kinase and focal adhesion kinase (FAK). This study establishes that HER2/HRG signaling selectively upregulates Tyr phosphorylation of c-Src at Tyr-215 located within the SH2 domain, increases c-Src kinase activity and selectively upregulates Tyr phosphorylation of FAK at Tyr-861. HER2-overexpressing tumors showed increased levels of c-Src phosphorylation at Tyr-215. These findings suggest that HER2/HRG influence metastasis of breast cancer cells through a novel signaling pathway involving phosphorylation of FAK tyrosine 861 via activation of c-Src tyrosine 215.

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Key words: Receptor tyrosine kinase; Cytoplasmic tyrosine kinase; Growth factor signaling

1. Introduction

Human epidermal growth factor receptor 2 (HER2/neu) [1], heregulin (HRG), a combinatorial HER ligand [2], and Src kinases [3,4] have been implicated in the regulation of breast cancer progression. Growth factor signaling regulates c-Src kinase activity by altering the tyrosine phosphorylation of specific Tyr residues. Tyrosine phosphorylation at residues Tyr-215 and Tyr-418 increases c-Src kinase activity, while Tyr phosphorylation at Tyr-529 down regulates c-Src kinase activity [4]. Focal adhesion kinase (FAK), a non-receptor tyrosine kinase and substrate of c-Src present in focal adhesions, is implicated in the regulation of cell motility, adhesion, and anti-apoptotic signaling [5,6]. Growth factors induce phosphorylation of FAK on a number of Tyr residues and each of the FAK Tyr residues has been implicated in generating a distinct signal. For example, FAK Tyr-397 in recruiting c-Src, phosphoinositide 3'-kinase and p130CAS to focal adhesions; FAK Tyr-576 and FAK Tyr-577 in upregulating FAK kinase activity [7]; FAK Tyr-925 in activating the Ras-mitogen-activated protein kinase pathway [8]; and FAK Tyr-861 in Ras-mediated transformation [9]. Cells lacking FAK are refractory

to platelet-derived growth factor- and epidermal growth factor-mediated motility signals [10].

Growth factor-mediated formation of motile structures involves regulation of FAK tyrosine phosphorylation [11]. Interestingly, HRG or activated HER2 reorganize the cytoskeleton, and increase the metastatic potential of breast cancer cells without an increase in the total tyrosine phosphorylation of FAK, suggesting HER2 might use a distinct pathway to regulate FAK [12,13]. However, very little information is known on the molecular mechanisms by which HER2 and HRG regulate Src and FAK signaling to alter the metastatic potential of breast tumor cells. The results from this study suggest that HRG and HER2 signaling selectively upregulates tyrosine phosphorylation of c-Src at Tyr-215 located within the SH2 domain, increases c-Src kinase activity and selectively upregulates tyrosine phosphorylation of FAK at Tyr-861.

2. Materials and methods

2.1. Cell cultures, reagents and plasmids

MCF-7 human breast cancer cells, NIH 3T3 cells, and B104 (NIH 3T3 cells expressing kinase-activated HER2) [13] were maintained in Dulbecco's modified Eagle's medium-F12 medium (1:1) supplemented with 10% fetal calf serum. Phospho-specific antibodies against FAK and Src were purchased from Biosource (Camarillo, CA, USA). Antibodies against HER2 (#MS325-P) and recombinant HRG β -1 were purchased from Neomarkers (Fremont, CA, USA). Antibodies against FAK (#F2918) and vinculin (#V913) were purchased from Sigma (St. Louis, MO, USA). c-Src WT cDNA expression vector (#21-114) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Src mutant Y215F was constructed using the site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) using the following primers: forward, AGCGGCGGTTTCTTCATCACCTCCCGACCCAG and reverse, CTGGGTGCGGGAGGTGATGAA-GAAACCGCGCT.

2.2. Cell extracts, immunoblotting and immunoprecipitation

MCF-7 cells were serum-starved for 48 h and treated with different concentrations of HRG (0.01 nM, 0.1 nM, or 1.0 nM). To prepare cell extracts, cells were washed three times with phosphate-buffered saline (PBS) and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 1× protease inhibitor cocktail (Roche Biochemical) and 1 mM sodium vanadate) for 15 min on ice.

2.3. Src kinase assays

In vitro Src kinase assays were performed following the previously described protocol [14]. Briefly, c-Src kinase was immunoprecipitated from 400 μ g of cell lysates treated with or without HRG. Immunoprecipitates were washed three times with Triton X-100 lysis buffer and three times with kinase buffer (20 mM HEPES pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol) and kinase reactions were performed using 10 μ g of acid-denatured enolase as a substrate at 30°C for 30

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Abbreviations: FAK, focal adhesion kinase; HER, human epidermal growth factor receptor; HRG, heregulin

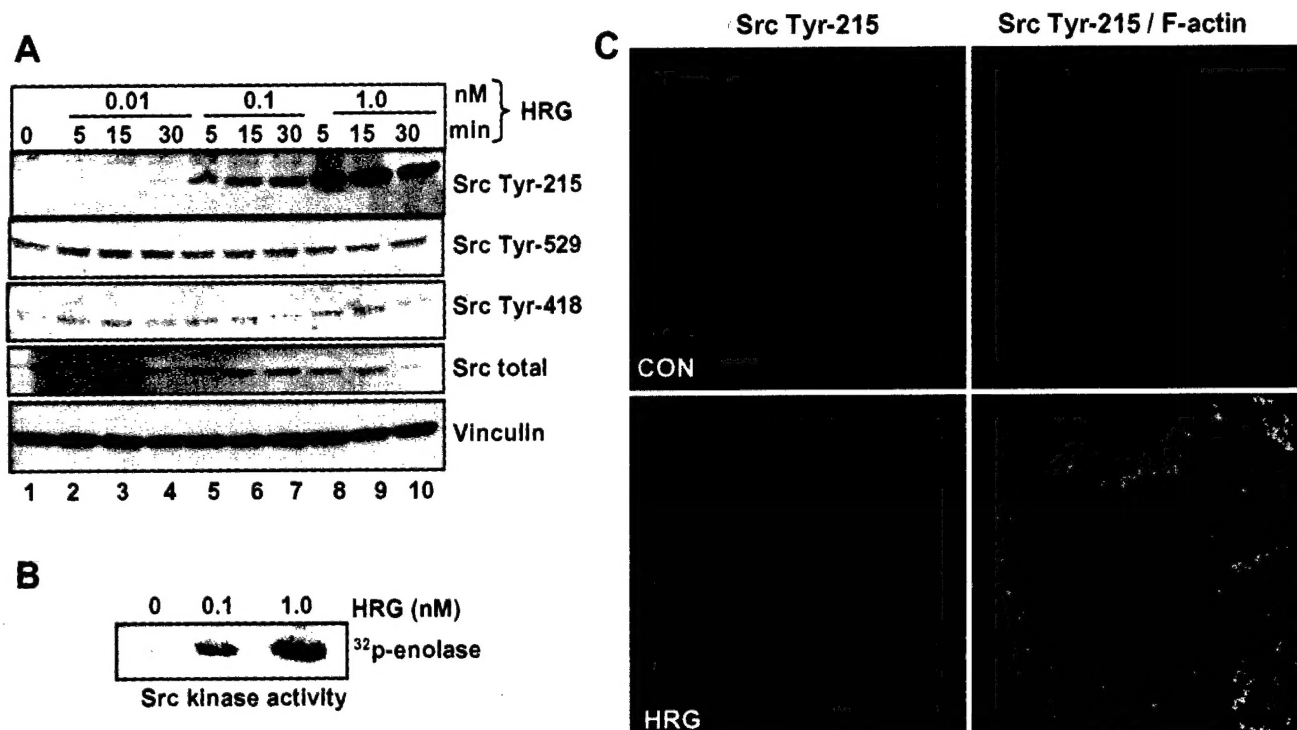


Fig. 1. HRG-induced Tyr phosphorylation of Src. A: Immunoblot analysis. MCF-7 cells were treated with 0.01 nM, 0.1 nM, or 1 nM HRG for the indicated times, and total lysates (200 μ g) were analyzed by immunoblotting with site-specific antibodies that recognize distinct Tyr sites on Src kinase. B: In vitro kinase analysis. MCF-7 cells were treated with or without HRG and kinase assays were performed using enolase as a substrate. C: Confocal microscopy analysis. Localization of Src Tyr-215 was studied in MCF-7 cells treated with or without HRG for 30 min. Src Tyr-215 is shown in green and phalloidin, a marker of F-actin-containing structures, is shown in red. Con indicates serum-starved MCF-7 cells not treated with HRG.

min, and visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography.

2.4. Immunofluorescence studies and confocal microscopy

For indirect immunofluorescence studies, cells were blocked by incubation with 10% normal goat serum in PBS for 1 h at ambient temperature. Cells were then incubated for 1 h at ambient temperature with polyclonal antibodies against FAK Tyr-861 or Src Tyr-215 and with monoclonal antibodies against vinculin. After four washes with PBS, cells were incubated with ALEXA-488- or fluorescein isothiocyanate-conjugated goat anti-mouse IgG or with ALEXA-546-conjugated goat anti-rabbit IgG (1:100 dilution, Molecular Probes, Eugene, OR, USA) in 10% normal goat serum (in PBS). Control cells were treated only with the secondary antibody. Slides were analyzed by confocal microscopy.

3. Results

3.1. HER2/HRG signaling uniquely upregulates Src phosphorylation at Tyr-215

To examine the possible participation of Src kinase in HER2/HRG signaling, we utilized three well-characterized phospho-specific antibodies [15–17], which uniquely recognize Tyr-215, Tyr-418 and Tyr-529 on Src. We used MCF-7 breast cancer cells, which are known to express HER2 and HER3. MCF-7 breast cancer cells were treated with HRG, a ligand that is known to activate the HER2 pathway [18,19]. HRG selectively enhanced c-Src Tyr-215 phosphorylation in a dose- and time-dependent manner (Fig. 1A). The highest level of phosphorylation was observed at a concentration of 1 nM, which was previously shown to elicit a migratory phenotype

in breast cancer cells [12]. Interestingly, there was no significant change in the phosphorylation of c-Src at Tyr-418, which was earlier linked to activation of Src kinase by a number of physiological signals [4]. Increased c-Src Tyr-215 phosphorylation correlated well with Src kinase activity as assessed by in vitro kinase assay (Fig. 1B). Confocal microscopy analysis of HRG-treated MCF-7 cells also demonstrated elevated c-Src Tyr-215 phosphorylation (Fig. 1C, bottom panel, green color). HRG treatment significantly induced the formation of motile structures such as ruffles, and importantly, a significant pool of activated c-Src Tyr-215 was localized in these structures. These results suggest that HRG may activate c-Src kinase via phosphorylation of Tyr-215 and that functionally activated c-Src kinase localizes to ruffles.

3.2. HRG induces phosphorylation of FAK at Tyr-861 in a c-Src kinase-sensitive manner

We next examined the functional consequence of HRG-induced stimulation of c-Src kinase by examining the phosphorylation status of a known downstream c-Src substrate, FAK. We used several commercially available Tyr site-specific antibodies against FAK Tyr sites [9]. We did not observe any significant increase in the phosphorylation of FAK at autophosphorylation site Tyr-397 (Fig. 2A). We also did not notice any increase in the Tyr phosphorylation of FAK Tyr-577, Tyr-925, rather, we observed a slight decrease in the level of tyrosine phosphorylation of these sites with an increase in HRG dose. Interestingly, HRG transiently stimulated the level of Tyr phosphorylation of FAK Tyr-861 in a dose-depen-

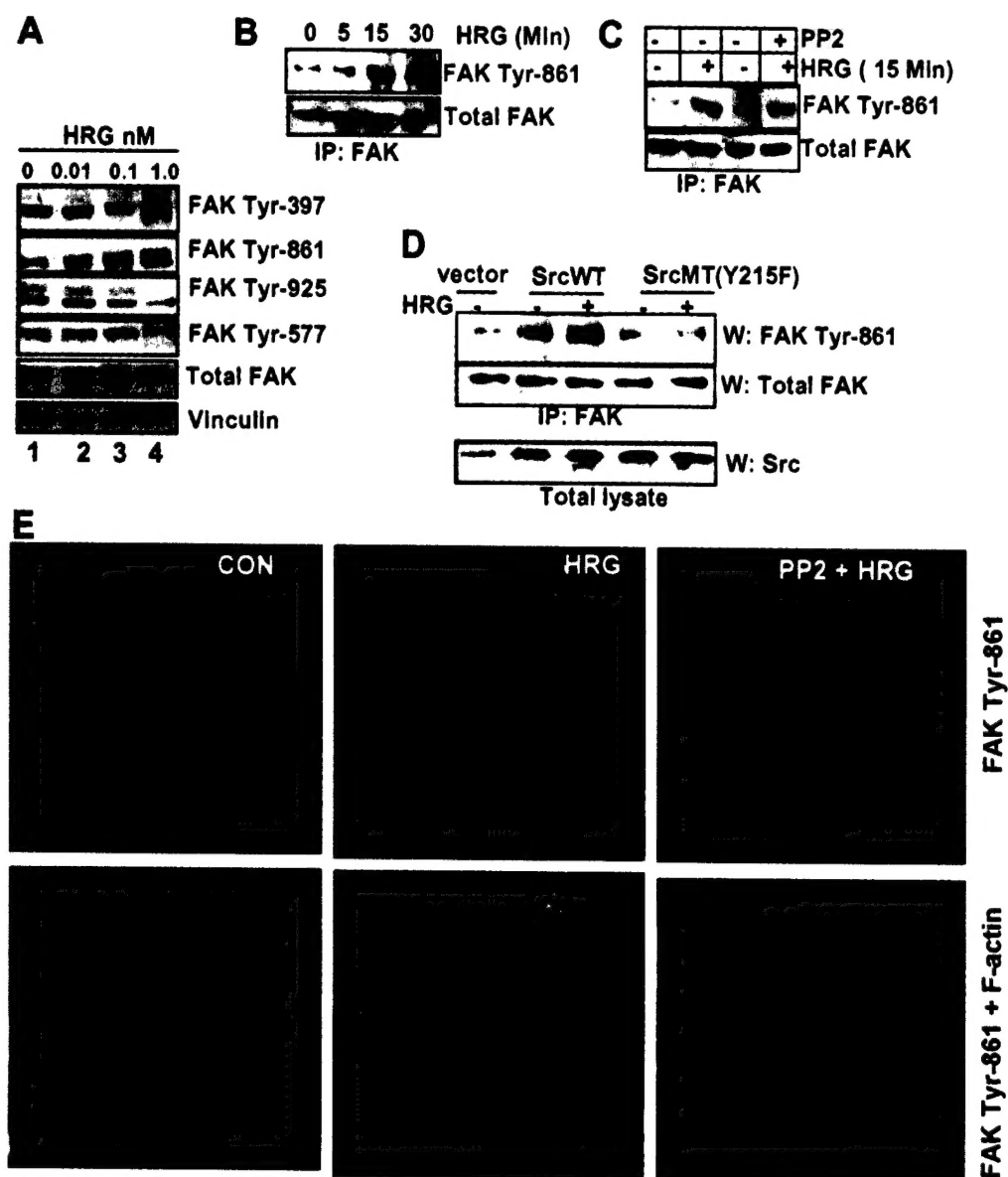


Fig. 2. HRG-induced tyrosine phosphorylation of FAK. **A:** Western blot analysis. MCF-7 cells were treated with 0.01 nM, 0.1 nM or 1 nM HRG for 30 min, and total lysates (200 μ g) were analyzed by Western blotting with site-specific antibodies that recognize distinct sites on FAK. **B:** MCF-7 cells were treated with 1 nM HRG for indicated periods of time, equal amount of protein was immunoprecipitated with FAK antibody. Blots were probed with phospho-specific antibody against FAK Tyr-861. Blot was stripped and reprobed with FAK antibody. **C:** MCF-7 cells were pretreated with or without the Src inhibitor PP2 for 30 min and then stimulated with 1 nM HRG for 15 min. Cell lysates were immunoprecipitated with FAK antibody, Western-blotted with phospho-specific FAK Tyr-861 antibody. **D:** MCF-7 cells were transfected with vector, Src wild type or Src mutant (SrcY215F) using Fugene-6 reagent in serum-free medium. After 24 h, cells were treated with 1 nM HRG for 15 min and equal amount of cell lysate was immunoprecipitated with FAK antibody followed by Western blotting with a phospho-specific antibody against FAK Tyr-861. Total lysate was run on a separate gel and analyzed by Western blotting using Src antibody (bottom panel) to visualize the expression of transfected Src constructs (lanes 2–5) compared to vector-transfected control (lane 1). **E:** Confocal microscopy analysis. Localization of FAK Tyr-861 was studied in MCF-7 cells treated with or without HRG for 30 min. As indicated some cells were pretreated with the Src inhibitor PP2 for 30 min before the addition of HRG. FAK Tyr-861 is shown in green, and phalloidin, a marker of F-actin-containing structures, is shown in red. Con indicates serum-starved MCF-7 cells not treated with HRG.

dent manner (Fig. 2A). HRG increased FAK Tyr-861 phosphorylation in a time-dependent manner with maximal activation at 15 min (Fig. 2B). The HRG-mediated increase in FAK phosphorylation at Tyr-861 was blocked by pretreatment of cells with the Src inhibitor PP2 (Fig. 2C). To mechanistically link HRG-mediated Src Tyr-215 phosphorylation to FAK Tyr-861 phosphorylation, we generated a c-Src construct in which tyrosine of Src at 215 was mutated to phenylalanine (Y215F). Using this mutant we analyzed HRG-mediated signaling to FAK at Tyr-861. The results suggest that

expression of wild type Src substantially increased both basal and HRG-mediated phosphorylation of FAK at Tyr-861 while expression of the Src Y215F mutant failed to increase HRG-mediated upregulation of phosphorylation of FAK at Tyr-861 (Fig. 2D). Confocal microscopy analysis also showed that HRG induced phosphorylation of FAK Tyr-861 (Fig. 2E, green color, middle panel) as compared to control (Fig. 2E, left panel). Furthermore, HRG-induced ruffle formation as well as FAK Tyr-861 phosphorylation was blocked by pretreatment of cells with the Src-specific inhibitor PP2 (Fig.

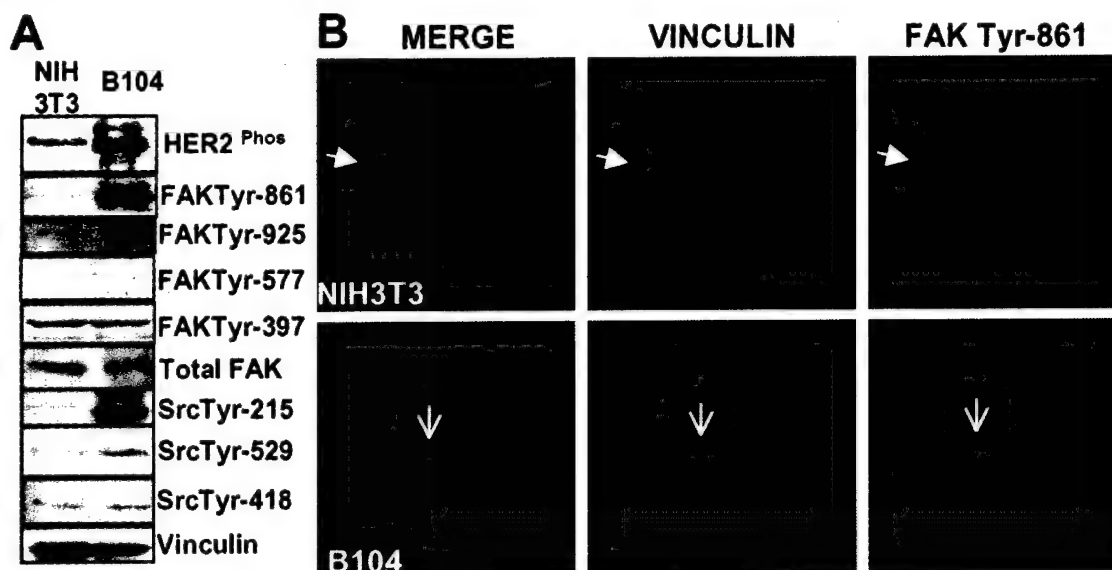


Fig. 3. Analysis of signaling pathways in NIH 3T3 cells expressing constitutively activated HER2. A: Western blot analysis. NIH 3T3 cells and B104 cells (NIH 3T3 cells expressing activated HER2) [13] were lysed and analyzed using phospho-specific antibodies against the indicated molecules. B: Confocal microscopy analysis. The status and localization of FAK Tyr-861 was studied in NIH 3T3 cells and B104 cells. FAK Tyr-861 is shown in red and vinculin, a marker of focal adhesions, is shown in green. Thick arrows point to localization of FAK Tyr-861 in dot-like focal adhesions in NIH 3T3 cells. Empty arrows point to the cytoplasmic localization of FAK Tyr-861.

2E, right panel) [20]. Together these results suggest that HRG-induced stimulation of FAK Tyr-861 phosphorylation might be mediated by c-Src kinase via its activation by phosphorylation at Tyr-215.

3.3. Activated HER2 modulates phosphorylation of Src at Tyr-215 and FAK at Tyr-861

We next analyzed the effect of HER2 overexpression on the status of Src Tyr-215 and FAK Tyr-861 using the NIH 3T3 cell line and a well-characterized NIH 3T3 cell line, B104, that expresses activated HER2 [13]. B104 cells exhibited elevated phosphorylation of c-Src Tyr-215 and FAK Tyr-861 compared to the phosphorylation in control NIH 3T3 cells. Minimal or no change was found in the status of tyrosine of FAK at Tyr-577, Tyr-925 and Tyr-397. To confirm the presence and

subcellular localization of FAK Tyr-861 phosphorylation in NIH 3T3 and B104 cells, we performed immunofluorescence studies followed by confocal microscopy. The results showed that despite a high level of FAK Tyr-861 phosphorylation in B104 cells, its location was primarily in the cytoplasm (Fig. 3B, thin arrows), as compared to a lower overall amount of phosphorylated FAK Tyr-861 distributed in a distinct dot-like pattern in the parental NIH 3T3 cells (Fig. 3B, thick arrows). Moreover, distinct differences in the cell surface morphology were also noticed (very well spread in NIH 3T3 cells vs. bipolar in B104 cells) and the amount of FAK Tyr-861 per cell surface unit was much greater in B104 cells than in parental NIH 3T3 cells. These findings suggest that active HER2 signaling mimics the events of HRG signaling including phosphorylation of c-Src Tyr-215 and FAK Tyr-861.

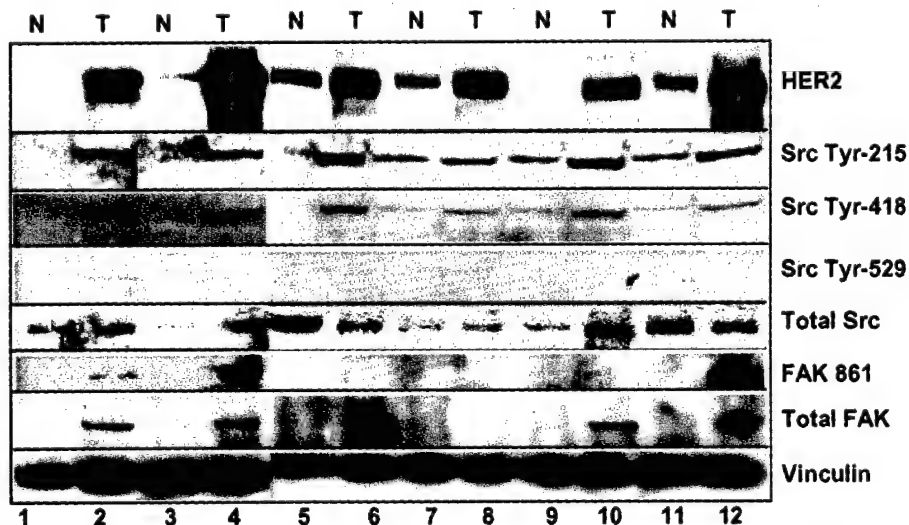


Fig. 4. Upregulation of tyrosine phosphorylation of Src at Tyr-215 in HER2-overexpressing breast tumors. Western blot analysis. Lysates from normal and breast tumor specimens were analyzed by immunoblotting with site-specific antibodies that recognize distinct Tyr sites on Src and FAK kinases. Blots were probed with anti-HER2 antibody to analyze the status of HER2 in tumors. Vinculin was used as a loading control.

3.4. Upregulation of Src Tyr-215 phosphorylation in breast tumors

To confirm the in vitro cell culture data, we analyzed the status of tyrosine phosphorylation of c-Src in a small number of breast tumor specimens. Of the six pairs of breast tumor samples analyzed, five tumors with high HER2 levels exhibited increased levels of tyrosine phosphorylation on Src Tyr-215 compared to tumors without HER2 overexpression or normal controls (Fig. 4). A modest increase in the level of Tyr-418 was also seen in tumors while little change in the Tyr-529 was observed. Results show the coexistence of increased levels of phosphorylation of Src at Tyr-215 and FAK at Tyr-861 in three of six HER2-overexpressing tumors (Fig. 4). These results provide proof of principle that the Src Tyr-215–FAK Tyr-861 pathway we identified in the MCF-7 cell culture model also occurs in breast tumors.

4. Discussion

Src kinase activity has previously been shown to be elevated in HER2-induced mammary tumors [21]. Our results suggest that Src kinase signaling might play a unique role in signaling by HRG and HER2. HRG activated c-Src via tyrosine phosphorylation of Tyr-215 located in the SH2 domain in a dose- and time-dependent manner and increased Src kinase activity. Furthermore, overexpression of constitutively active HER2 also induced phosphorylation of c-Src Tyr-215. Activated c-Src Tyr-215 distinctly localized to actin-containing motile structures such as ruffles in HRG-treated MCF-7 cells and in B104 cells which express activated HER2. In the literature, we could find only one other report showing phosphorylation of Src Tyr-215 by a growth factor, platelet-derived growth factor, and such phosphorylation was reported to result in activation of Src kinase due to disruption of SH2 binding to the C-terminal region of c-Src [22]. Our results support the earlier observation of tyrosine phosphorylation of c-Src at Tyr-215 by growth factors and further suggest that c-Src Tyr-215 phosphorylation may have a unique effect on the localization of c-Src and its substrate specificity.

Despite an enhancement of the c-Src kinase activity by Tyr phosphorylation at Tyr-215 by HRG, only selective change in the phosphorylation of the Src substrate FAK at Tyr-861 was observed. Earlier studies demonstrated that FAK Tyr-861 is a major site of phosphorylation by Src kinase [23]. Vascular endothelial growth factor signaling-mediated endothelial cell migration and anti-apoptosis functions were shown to involve Tyr phosphorylation of FAK at 861 via Src kinase [18]. Recently, phosphorylation of FAK at Tyr-861 was shown to correlate with Ras-induced transformation of fibroblasts [9]. The results of this study suggest that FAK phosphorylation at Tyr-861 also represents a mechanism by which both HRG and activated HER2 may influence the malignant phenotype of epithelial cells since blockage of Src kinase activity by a selective inhibitor PP2 resulted in the reduction of ruffle formation induced by HRG. The findings that HRG and HER2 activated Src kinase and that the Src inhibitor PP2 blocked both the upregulation of FAK Tyr-861 phosphorylation and HRG-mediated cytoskeletal changes suggest that the Src Tyr-215 to FAK Tyr-861 pathway may be important for HRG and HER2 signaling to mediate cytoskeletal changes.

c-Src is also known to utilize Tyr-397, an autophosphory-

lation site, to associate with FAK [6]. Since HRG did not cause any changes in the phosphorylation of FAK Tyr-397, HRG- and HER2-activated c-Src may interact with FAK via other interacting sites, and selectively phosphorylate c-Src at Tyr-861. In this context, v-Src was previously shown to induce FAK Tyr phosphorylation, independently of Tyr-397 phosphorylation [24]. Identification of HRG signaling to FAK kinase via phosphorylation of the SH2 domain raises the possibility that the phospho antibodies against c-Src Tyr-215 may serve as a prognostic marker. However, a larger study would need to be conducted to establish the significance of these tools. In summary, our results suggest that HER2 and HRG systems uniquely regulate signaling from focal adhesion complexes through selective phosphorylation of c-Src Tyr-215 and FAK Tyr-861.

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Differential Regulation of Components of the Focal Adhesion Complex by Heregulin: Role of Phosphatase SHP-2

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Heregulin (HRG) has been implicated in the progression of breast cancer cells to a malignant phenotype, a process that involves changes in cell motility and adhesion. Here we demonstrate that HRG differentially regulates the site-specific phosphorylation of the focal adhesion components focal adhesion kinase (FAK) and paxillin in a dose-dependent manner. HRG at suboptimal doses (0.01 and 0.1 nM) increased adhesion of cells to the substratum, induced phosphorylation of FAK at Tyr-577, -925, and induced formation of well-defined focal points in breast cancer cell line MCF-7. HRG at a dose of 1 nM, increased migratory potential of breast cancer cells, selectively dephosphorylated FAK at Tyr-577, -925, and paxillin at Tyr-31. Tyrosine phosphorylation of FAK at Tyr-397 remained unaffected by HRG stimulation. FAK associated with HER2 only in response to 0.01 nM HRG. In contrast, 1 nM HRG induced activation and increased association of tyrosine phosphatase SHP-2 with HER2 but decreased association of HER2 with FAK. Expression of dominant-negative SHP-2 blocked HRG-mediated dephosphorylation of FAK and paxillin, leading to persistent accumulation of mature focal points. Our results suggest that HRG differentially regulates signaling from focal adhesion complexes through selective phosphorylation and dephosphorylation and that tyrosine phosphatase SHP-2 has a role in the HRG signaling. *J. Cell. Physiol.* 190: 189–199, 2002. © 2002 Wiley-Liss, Inc.

Growth factors and their receptors play an essential role in regulating epithelial cell proliferation, and perturbation in the regulated expression or function of growth factors may contribute to the progression and maintenance of breast cancer. For example, human epidermal growth factor receptor (HER2) overexpression is frequently associated with an aggressive clinical course, short disease-free survival, poor prognosis, and increased metastasis in human breast cancer (Slamon et al., 1987; Reese and Slamon, 1997). In addition, progression of human breast cancer cells may be regulated by heregulin (HRG) a combinatorial ligand for HER3 and HER4 (Tang et al., 1996). The regulation of HER family members is complex, as they can be transactivated by heterodimeric interactions between HER members and thus can utilize multiple signaling pathways to execute their biological functions. For example HRG bound HER3 or HER4 can activate HER2 receptor as a result of HER2/HER3 or HER2/HER4 heterodimeric interactions (Graus-Porta et al., 1997). Recently, we as well as others have demonstrated that HRG activation of breast cancer cells promotes the development of more aggressive phenotypes (Adam et al., 1998; Aguilar et al., 1999). The activation of HRG-signaling pathways has also been linked to the progres-

sion of breast cancer cells to a more invasive phenotype (Sepp-lorenzino et al., 1996; Vadlamudi et al., 1999a,b). These observations suggest that both ligand-driven activation of HER and constitutive HER activation could play important roles in the progression of breast cancer cells to a malignant phenotype.

One of the earliest responses of cells to extracellular growth factors is rapid reorganization of their cytoske-

Abbreviations: HRG, heregulin-beta1; FAK, focal adhesion kinase; Tyr, tyrosine; HER, human epidermal growth factor receptor; SHP-2, SH2 domain-containing protein-tyrosine phosphatase 2.

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letons and cell shapes. In addition, cell transformation and invasiveness require, among other steps, changes in cell motility and adhesion that are regulated by the sequential formation and dissolution of focal adhesion complexes, which are the points of contact between the substrate and the cells (Burrige and Chrzanowska-Wodnicka, 1996). Focal adhesion kinase (FAK) is one of the well-characterized protein in focal adhesion complexes, and it has been implicated in the regulation of cell motility, adhesion, and anti-apoptotic signaling (Sieg et al., 1999). For example, overexpression of FAK leads to increased cell migration of Chinese hamster ovary (CHO) cells (Cary et al., 1996), and conversely, suppression of FAK by a dominant-negative mutant reduces the migratory potential of CHO cells (Gilmore and Romer, 1996). FAK is also shown to have a role in prostate carcinoma cell migration (Zheng et al., 1999). FAK-null fibroblasts exhibit a round morphology, defects in cell migration, and more focal adhesions (Sieg et al., 1999). FAK-deficient mice are embryonic-lethal; however, mesodermal cells derived from these embryos show decreased cell spreading and motility (Ilic et al., 1995). FAK is also overexpressed (Owens et al., 1995) and amplified in several human cancers (Agochiya et al., 1999). Engagement of integrins and other adhesion receptors can induce activation of FAK (Burrige and Chrzanowska-Wodnicka, 1996), which leads to phosphorylation of several tyrosine residues through autophosphorylation, recruitment of the cytoplasmic tyrosine kinase Src (Sieg et al., 1999), or cell-surface receptors (Zachary, 1997). Each of the FAK tyrosine residues is implicated in generating a distinct signal, FAK Tyr-397 in recruiting Src, PI-3 kinase and p130CAS to focal adhesions; FAK Tyr-576 and -577 in upregulating FAK-kinase activity (Ruest et al., 2000) and FAK Tyr-925 in activating the Ras-MAPK pathway (Schlaepfer and Hunter, 1997); the functions of FAK Tyr-407 and -861 are yet to be established (Calalb et al., 1996). However, very little information is available on how HER2 or HRG might use FAK to alter the metastatic potential of breast tumor cells.

Growth factor stimulation also leads to a rapid increase in tyrosine phosphorylation of the focal adhesion protein paxillin. The activation of focal adhesion complexes then initiates a cascade of interactions with other proteins containing SH2/SH3 domains (Src, v-Crk, and vinculin) or with the components of Ras signaling (Grb2 and Sos) (Schlaepfer et al., 1994; Bergman et al., 1995). FAK and paxillin are phosphorylated on tyrosine residues by a number of growth factors, including platelet derived growth factor (Abedi et al., 1995), epidermal growth factor (Sieg et al., 2000) vascular endothelial growth factor (Abedi and Zachary, 1997), insulin like growth factor-1 (Leventhal et al., 1997), and hepatocyte growth factor (Matsumoto et al., 1994). Tyrosine phosphorylation of paxillin on Tyr-31 and -118 is stimulated upon cell adhesion, and to create binding sites for the adaptor protein Crk (Bellis et al., 1995). FAK has been implicated in phosphorylating paxillin at these sites, either directly (Bellis et al., 1995) or indirectly by recruiting Src family of tyrosine kinases (Matsumoto et al., 1994; Thomas et al., 2000).

Despite the well-characterized roles of FAK and paxillin in focal adhesion formation, the functions of

these signaling components in the actions of HRG remain unknown. The present study was designed to determine the nature of the early signaling events in focal adhesion complex formation that may be stimulated by HRG. Here we report that HRG differentially regulates the components of focal adhesion complexes by selectively phosphorylating and dephosphorylating distinct tyrosine residues and by modulating interactions among the HER family receptors.

MATERIALS AND METHODS

Cell cultures and reagents

MCF-7 human breast cancer cells (Adam et al., 1998), and MCF-7 C/S #14 cells (expressing dominant-negative SHP-2 C/S) (Manes et al., 1999) were maintained in DMEM-F12 (1:1) supplemented with 10% fetal calf serum. Phosphospecific antibodies against FAK and paxillin were purchased from Biosource International (Camarillo, CA). Antibodies against HER2 (#MS325-P), PY20 (#MS445-P), paxillin (#MS404-P), and recombinant HRG beta-1 were purchased from Neomarkers, Inc. (Fremont, CA). Antibodies against FAK (#F2918) and vinculin (#V913) were purchased from Sigma (St. Louis, MO). Phospho p42/44 (#9105S), phospho Akt, and p38^{MAPK} (#9211S) were purchased from New England Biolabs (Boston, MA). Antiphosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY).

Cell migration and adhesion assays

Cell migration assays were performed using modified Boyden chambers assay (Vadlamudi et al., 1999a,b). Serum starved MCF-7 cells were trypsinized and loaded into the upper well of Boyden chamber (20,000 cells/well). The lower side of separating filter was coated with a thick layer of 1:1 diluted Matrigel (Life Technologies, Inc., Gaithersburg, MD) in serum free medium. The number of cells that successfully migrated through the filter and invaded the Matrigel as well as cells that remained on the upper side of the filter were counted by confocal microscopy after staining with propidium iodide (Sigma). Results were expressed as percentage of migrated cells compared with total number of cells. For cell adhesion assays, cells were detached with PBS-5 mM EDTA solution and plated into collagen I or collagen IV coated Cytomatrix cell adhesion strips (Chemicon International, Inc., Temecula, CA). The cells were pretreated with various doses of HRG before plating and incubated for 30 min at 37°C. The cells were rinsed with PBS, stained with 0.2% crystal violet in 10% ethanol for 5 min. Cells were washed three times with PBS. The attached cells were then solubilized for 5 min with 1:1 mixture of 0.1 M NaH₂PO₄, pH 4.5 and 50% ethanol and absorbency was measured at 570 nm using a microplate reader. Cellular adhesion was reported as a percentage of that observed with control MCF-7 cells which were not treated with HRG.

Cell extracts, immunoblotting, and immunoprecipitation

MCF-7 cells were serum starved for 48 h and treated with different concentrations of HRG (0.01, 0.1, 1.0 nM). To prepare cell extracts, cells were washed three times

with phosphate buffered saline (PBS) and then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 1× protease inhibitor cocktail (Roche Molecular Biochemicals Indianapolis, IN) and 1 mM sodium vanadate) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates containing equal amounts of protein (~200 µg) were resolved on SDS-polyacrylamide gels (10% acrylamide), transferred to nitrocellulose membranes, probed with the appropriate antibodies, and developed using either enhanced chemiluminescence method or the alkaline phosphatase-based color reaction method. For immunoprecipitation of HER family members, cells were lysed with NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% NP-40, 1× protease inhibitor cocktail, 1 mM sodium vanadate). Immunoprecipitations were performed for 2 h at 4°C using 1 µg of antibody per mg of protein.

Phosphatase assays

Tyrosine phosphatase assays were performed using nonradioactive tyrosine phosphatase assay kit as per manufacturer's instructions (Boehringer Mannheim, Germany). This assay involves uses of synthetic phosphotyrosine containing peptides coated to a microtiter plate. MCF-7 cells were treated with different doses of HRG and cells were lysed with RIPA buffer. Lysates were diluted with RIPA buffer 1:200 and 5 µl was incubated in the microtiter plates for 30 min at 37°C in 60 µl of reaction buffer. Reaction was quenched by addition of 100 µM sodium vanadate. The fraction of unmetabolized substrate is determined by immunochemistry using antiphosphotyrosine antibodies conjugated to peroxidase and addition of substrate from the kit. Absorbency of the sample was measured at 405 nm using a microtiter plate reader. Phosphatase activity was expressed as the percentage of activity in the control untreated cells.

Immuno-fluorescence and confocal microscopy

For indirect immunofluorescence, cells were blocked by incubation with 10% normal goat serum in PBS for 1 h at ambient temperature. Cells were then incubated for 1 h at ambient temperature with polyclonal antibodies (pAb) against FAK Tyr-925, FAK Tyr-577 or paxillin Tyr-31 and with vinculin monoclonal antibody (mAb). After four washes with PBST, cells were incubated with ALEXA-488 or FITC-conjugated goat anti-mouse IgG or ALEXA-546 conjugated goat anti-rabbit IgG (Molecular Probes) (1:100 dilution) in 10% normal goat serum (in PBS). For controls, cells were treated only with the secondary antibody. Slides were analyzed by confocal microscopy.

³²P-labeling

MCF-7 cell were in vivo equilibrium labeled with [³²P]-orthophosphoric acid for 10 h and treated with HRG. SHP-1 and -2 were immunoprecipitated and separated by SDS-PAGE and phosphorylation was visualized by autoradiography with phosphorimager.

RESULTS

HRG regulates tyrosine phosphorylation of FAK and paxillin in a dose dependent manner

To determine the nature of early signaling events during HRG stimulation of breast cancer cells, we initially evaluated the effects of various doses of HRG on the migratory potential of noninvasive breast cancer MCF-7 cells. Cell migration assays were performed using modified Boyden chamber assay as described in the Materials and Methods section. MCF-7 cells exhibited very little migratory potential and HRG at 0.1 and 1 nM increased the migratory potential with highest migration at 1 nM. Low dose of HRG (0.01 nM) has very little effect on the migratory potential (Fig. 1A). In earlier studies we observed that HRG also induces scattering of MCF 7 cells when plated on an extracellular matrix collagen (Vadlamudi et al., 1999a,b). Since scattering and cell migration involves changes in the cell adhesion, we then measured the effects of doses of HRG on the adhesion properties of MCF-7 cells using purified extracellular matrix proteins collagen I and IV. Low concentration of HRG (0.01 nM) significantly increased the adhesion of MCF-7 cells to the matrix while high concentration (1 nM) has little or no effect on the adhesion (Fig. 1B). Since HRG at 1 nM substantially increased the migratory potential of MCF-7 cells, we designated 1 nM HRG as an optimal dose for migration and 0.01 nM as a suboptimal dose as it had very little or no effect on the cell migration.

Since focal adhesion complexes play an important role in the modulation of cell migration, we next analyzed dose effects of HRG on the regulation of two important signaling proteins in focal adhesions FAK and paxillin. Cell lysates from control or HRG treated cells were immunoprecipitated with anti-FAK or anti-paxillin antibody and blotted with phosphotyrosine antibody. HRG stimulated tyrosine phosphorylation of FAK and paxillin at suboptimal doses (0.01, 0.1 nM) but dramatically reduced the tyrosine phosphorylation at higher dose (1.0 nM) (Fig. 1C). Reduction in the tyrosine phosphorylation appears due to dephosphorylation rather than changes in the kinetics since we failed to see any increase in the tyrosine phosphorylation at shorter time intervals (Fig. 1D).

HRG regulates FAK and paxillin phosphorylation on specific residues

FAK can be tyrosine phosphorylated on a number of tyrosine residues, including Tyr-397, -925, -577 in response to various stimuli (Schlaepfer and Hunter, 1998; Ruest et al., 2000). To map HRG-responsive phosphorylation sites on FAK, we employed a series of well-characterized phosphospecific antibodies (Ruest et al., 2000; Sieg et al., 2000; Vial et al., 2000). HRG at a dose of 0.01 nM transiently stimulated Tyr-577 phosphorylation (Fig. 2A); however, this site showed very low or no tyrosine phosphorylation at 1 nM HRG. Low doses of HRG did not affect phosphorylation of Tyr-925, while 1 nM HRG caused significant dephosphorylation at this site (Fig. 2A). HRG had little or no effect on the phosphorylation of Tyr-397.

Paxillin is phosphorylated on Tyr-31 and -118 in response to adhesion to fibronectin (Bellis et al., 1995).

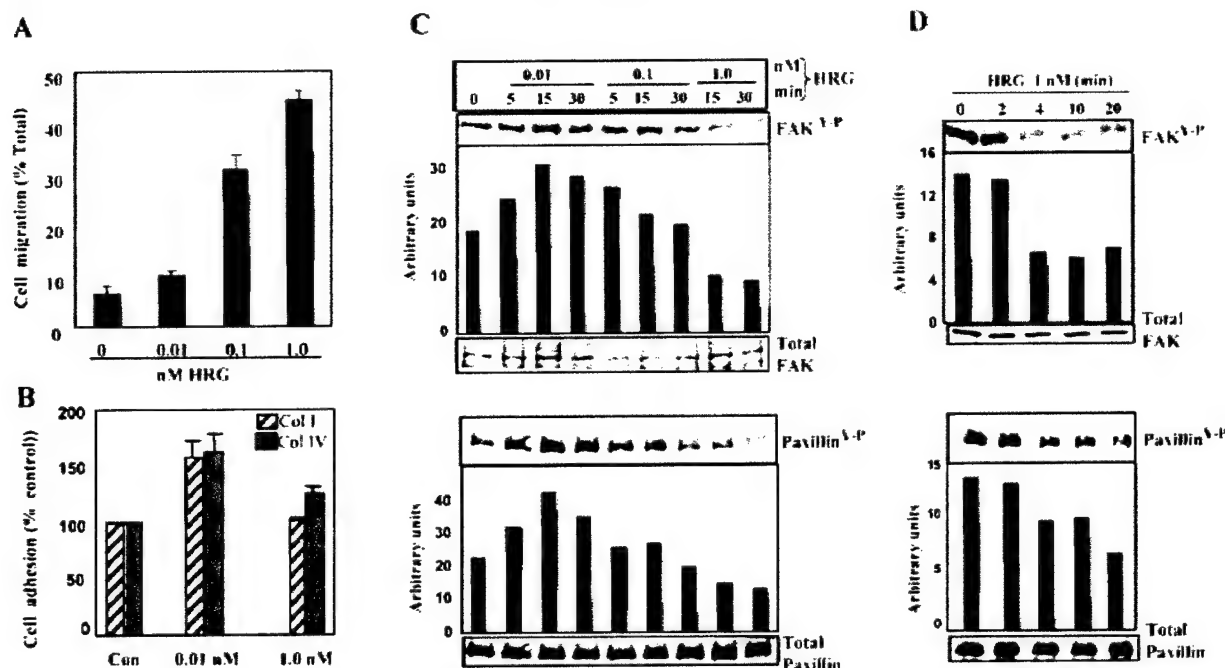


Fig. 1. Dose dependent effects of HRG on cell migration and adhesion. A: Effect of various doses of HRG on cell migration as determined using modified Boyden chamber assay. Results shown are representative of three separate experiments. B: Effect of low (0.01 nM) and high (1.0 nM) dose of HRG on cell adhesion on wells coated with either collagen I or collagen IV. Data shown are means of triplicate wells and are representative of two independent experiments. Adhesion was measured 30 min after incubation. C,D: HRG

induces dephosphorylation of FAK and paxillin in a dose dependent manner. MCF-7 cells were treated with 0.01, 0.1, or 1 nM HRG for indicated times, and equal amounts of cell lysates were immunoprecipitated with antibodies against FAK or paxillin and immunoblotted with antibodies against phosphotyrosine, FAK or paxillin. Intensity of the phosphotyrosine bands were quantitated by the SIGMA scan program and shown as a graph with arbitrary units.

Since we observed a reduction of total tyrosine phosphorylation of paxillin at 1 nM HRG, we examined the effect of HRG on Tyr-31. Similar to its affect on FAK, 0.01 nM HRG stimulated Tyr-31 phosphorylation of paxillin, but 1 nM HRG reduced the level of Tyr-31 phosphorylation (Fig. 2B). Together, these results suggested a biphasic response to HRG on specific sites of FAK and paxillin.

HRG regulation of FAK and paxillin tyrosine phosphorylation in vivo

To confirm the significance of HRG-mediated changes in the tyrosine phosphorylation of FAK and paxillin, we examined the existence of these events in vivo. MCF-7 cells were treated with 0.01 nM or 1 nM HRG for 15 min and FAK and paxillin phosphorylation were analyzed by dual labeling immunofluorescence using a mouse mAb against vinculin (as a marker of focal adhesions, green color) and rabbit pAb against phosphorylated forms of FAK or paxillin (red color, Fig. 3A). In control cells, immunostaining of FAK Tyr-577 and -925, and paxillin Tyr-31 was predominantly co-localized with vinculin containing focal adhesion complex dots (Fig. 3, upper panel); however, 0.01 nM HRG increased staining for all three sites (Fig. 3, middle panel) while 1 nM HRG caused a dramatic loss of staining intensity (Fig. 3, lower panel). Analysis of the morphology of the focal contacts revealed that at suboptimal doses (0.01 nM), HRG-activated cells were anchored to the substratum by mature focal

adhesion points, represented by long, stripe-like shapes at the periphery of each unpolarized cell. In contrast, when the cells are activated with optimal doses of HRG (1 nM), small focal adhesion points accumulated at one pole of the cell, corresponding to its leading edge, could be visualized exclusively by the vinculin staining. These points represent very dynamic, immature focal adhesion sites reminiscent of a motile cell phenotype (Fig. 3A-C, lower panels).

HRG activates distinct subsets of HER in a dose-dependent manner

We next examined the temporal relationship between FAK and paxillin tyrosine phosphorylation and the signaling pathways activated by HRG. HRG activates several signaling pathways including the $p42^{\text{MAPK}}$, $p38^{\text{MAPK}}$ and PI-3 kinase pathways (Sepp-Lorenzino et al., 1996; Vadlamudi et al., 1999a,b). We therefore analyzed the activation of signaling components (via HRG) using phosphospecific antibodies. As shown in Figure 4A, HRG enhanced the phosphorylation of $p42^{\text{MAPK}}$ and Akt (as a marker of PI-3 kinase activation) in a dose-dependent manner, with highest activation at 1 nM HRG, however $p42^{\text{MAPK}}$ was only transiently activated at 0.01 nM HRG. $p38^{\text{MAPK}}$ was only activated at 1 nM HRG.

Since all three signaling pathways were highly active at 1 nM HRG, we hypothesized that some of the observed dose-dependent effects were due to formation of distinct

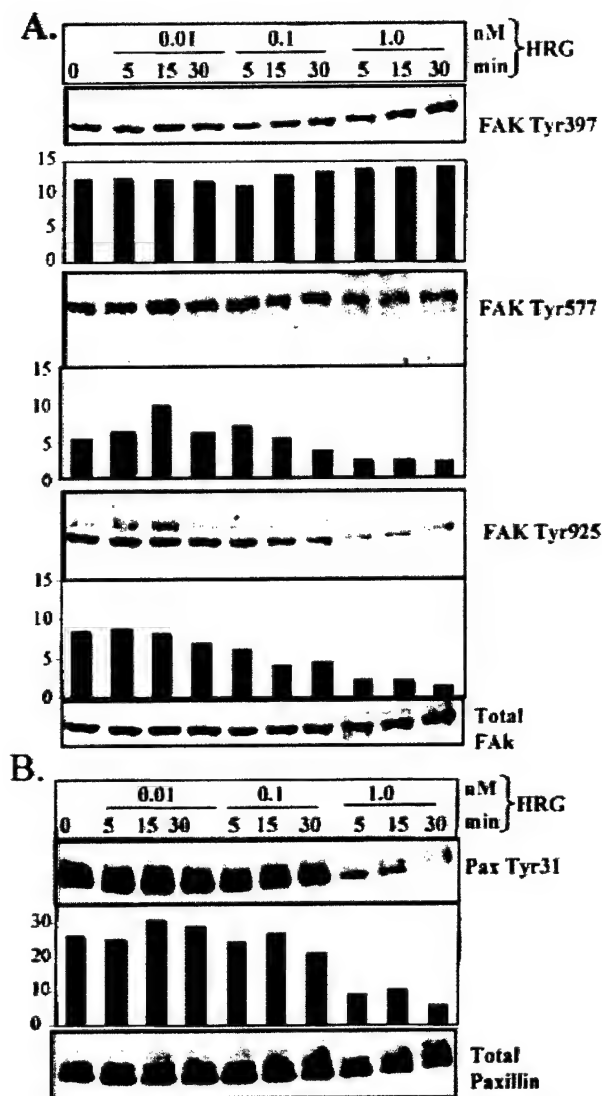


Fig. 2. HRG differentially regulates tyrosine phosphorylation of selective residues on FAK and paxillin in a dose-dependent manner. MCF-7 cells were serum-starved and treated with 0.01, 0.1 or 1 nM HRG for 30 min, and cell lysates were analyzed by immunoblotting with phosphotyrosine specific antibodies against FAK (A), and paxillin (B). Blots were stripped and reprobed with antibodies, which recognize total FAK and paxillin. Intensity of the bands were quantitated by the SIGMA scan program and shown as a graph (bottom panels).

complexes among the HER family members. HRG binds HER3 and HER4, and functional transduction of signaling depends on the formation of dimers with other members of the HER family and their transphosphorylation (Gamett et al., 1997). MCF-7 cells were treated with different doses of HRG, four HER members were immunoprecipitated using specific mAbs, and the tyrosine phosphorylation of each receptor was analyzed by blotting with anti-tyrosine mAb (Fig. 4B). The optimal dose of HRG predominantly increased the phosphorylation of HER2 and HER3, and 0.01 and 0.1 nM HRG significantly increased the tyrosine phosphorylation of

HER1 and HER2. An increase in HER4 phosphorylation was also observed at 1 nM HRG; however its intensity was much weaker than that of HER2 and HER3 phosphorylation (Fig. 4B). These results suggested that at a suboptimal HRG dose, signaling events were generated via EGFR/HER2 complexes. At an optimal dose, signaling events may have been generated primarily by the formation of HER2/HER3 complexes and possibly from HER4/HER2 heterodimers, which may play a role in tyrosine phosphorylation of FAK and paxillin. Since 1 nM HRG promoted a preferential downregulation of FAK and paxillin phosphorylation, the formation of HER2/HER3 complexes was further confirmed by immunoprecipitating HER3 and by blotting with an anti-HER2 mAb (Fig. 4C).

High doses of HRG stimulate phosphatase activity

Our results suggested that all signaling pathways analyzed were stimulated in cells treated with 1 nM HRG but our results did not explain the reduced tyrosine phosphorylation of FAK and paxillin at this dose. We therefore hypothesized that optimal doses of HRG activate a phosphatase, that dephosphorylates FAK and paxillin. As shown in Figure 5A, pretreatment of MCF-7 cells with the general tyrosine phosphatase inhibitor sodium vanadate blocked the 1 nM HRG-mediated dephosphorylation of FAK. To determine if HRG induces tyrosine phosphatase activity, we have used tyrosine phosphatase assay kit as described in experimental procedures. Direct determination of phosphatase activity in HRG-treated cells indicated that 1 nM HRG significantly increased the phosphatase activity over control (Fig. 5B).

Data from the literature suggest that SH2 domain-containing protein-tyrosine phosphatases SHP-1 and -2 associate with HER receptors (Vogel et al., 1993; Tomic et al., 1995), and that SHP-2 can dephosphorylate FAK and paxillin (Ouwens et al., 1996). To explore the potential involvement of these phosphatases in HRG-mediated dephosphorylation of FAK and paxillin, we analyzed the effect of HRG on the phosphorylation status of these phosphatases by immunoprecipitating lysates from MCF-7 cells treated with HRG and blotting with anti-phosphotyrosine antibody (Fig. 5D). Tyrosine phosphorylation of SHP-2 has been correlated with its activation (Vogel et al., 1993). Here we found that optimal dose of HRG (1 nM) stimulated tyrosine phosphorylation of SHP-2, but HRG has no effect on SHP-1 phosphorylation. To analyze the observed effect of HRG on SHP-2 phosphorylation in vivo, cells were metabolically labeled with ^{32}P -orthophosphate, and treated with different doses of HRG. SHP-1 and -2 were precipitated, and their phosphorylation was analyzed by autoradiography (Fig. 5C). HRG induced the phosphorylation of SHP-2 but not of SHP-1 in a dose-dependent manner. These results indicated that higher doses of HRG activated the phosphorylation of SHP-2.

HRG induces formation of distinct HER2-containing complexes in a dose-dependent manner

HER2 is the preferred heterodimer partner for HRG (Graus-Porta et al., 1997). Since FAK interacts with

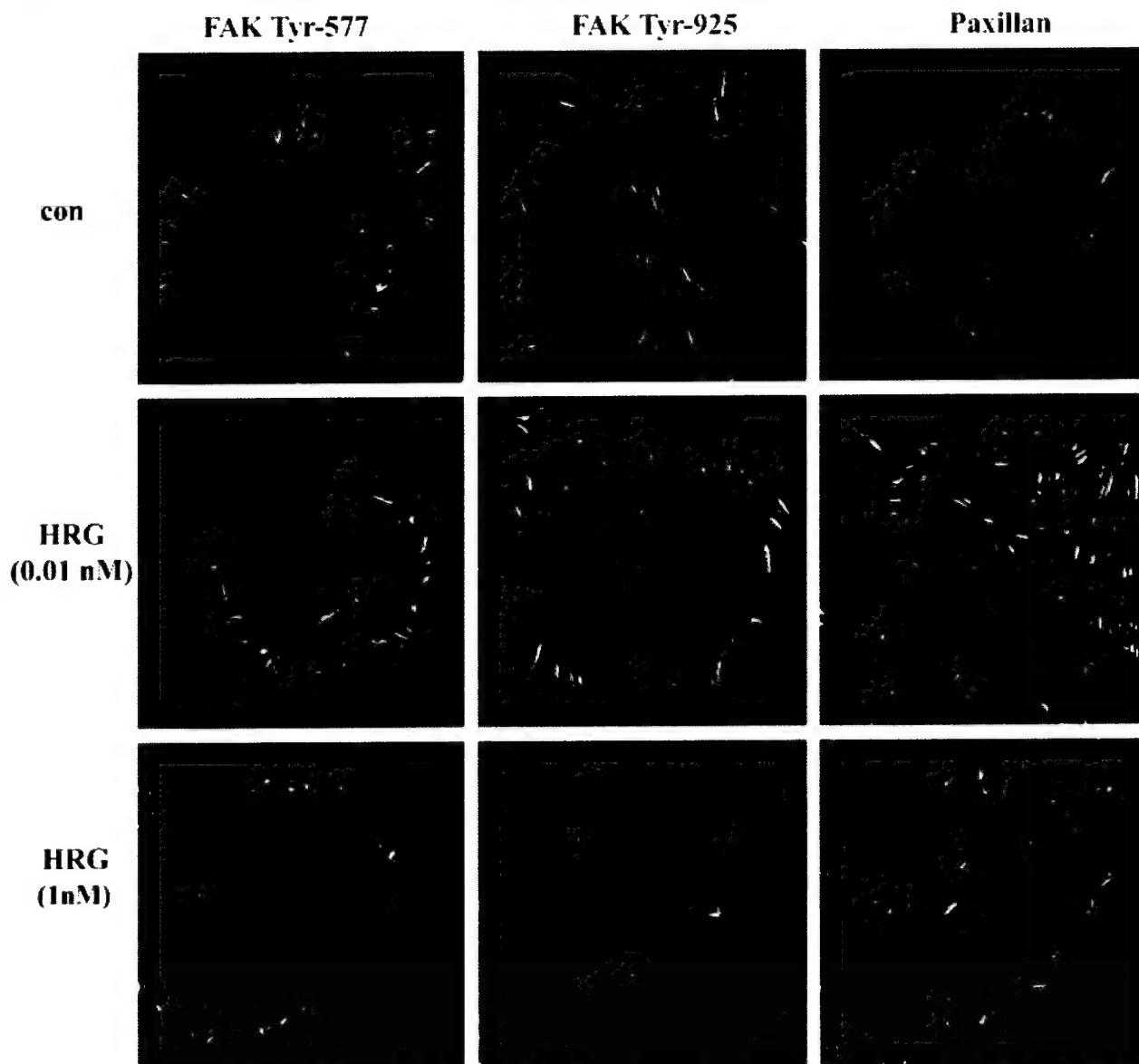


Fig. 3. HRG dose affects the status and localization of FAK and paxillin. MCF-7 cells were treated with 0.01 or 1 nM HRG for 30 min, and FAK and paxillin were analyzed by confocal microscopy after dual-labeling immunofluorescence using a mAb against vinculin (green color, as a marker of focal adhesions) and rabbit pAb against FAK Tyr-577 and Tyr-925, and paxillin Tyr-31 (red color). Yellow color indicates co-localization of vinculin with FAK or paxillin. Note that in control serum-starved cells (**upper panels**), all the FAK Tyr-577 and

Tyr-925, and paxillin Tyr-31 staining co-localized predominantly to vinculin-containing dots. At low doses of HRG (**middle panels**), cells were anchored to the substrate by mature focal adhesion points. At a high HRG dose, there was a dramatic loss of staining intensity corresponding to phosphorylated forms of FAK Tyr-577 and Tyr-925 or paxillin Tyr-31 (**lower panels**). At a high dose of HRG, cells displayed dynamic, immature dot-like focal adhesion sites reminiscent of a motile cellular phenotype.

HER2 and HER3 in Schwann cells (Vartanian et al., 2000) and because SHP-2 interacts with HER2 (Vogel et al., 1993), we examined the formation of HER2-containing complexes initiated by HRG. As shown in Figure 6A,B, 0.01 and 0.1 nM HRG, but not 1 nM HRG, promoted the association of FAK with HER2, as revealed by immunoprecipitation with either FAK or HER2 mAbs. In contrast, the association of SHP-2 with HER2 was preferentially enhanced only at 1 nM HRG (Fig. 6C,D).

Dominant-negative SHP-2 blocks HRG-induced dephosphorylation of FAK

Because of the increase in tyrosine phosphorylation and association of SHP-2 with HER2 at a higher concentration of HRG, we hypothesized that SHP-2 plays a role in HRG-mediated FAK Tyr-577 and paxillin Tyr-31 dephosphorylation. To examine this possibility, we used a well-characterized MCF-7 stable cell line that expressed SHP-2 C/S, a dominant-negative mutant of

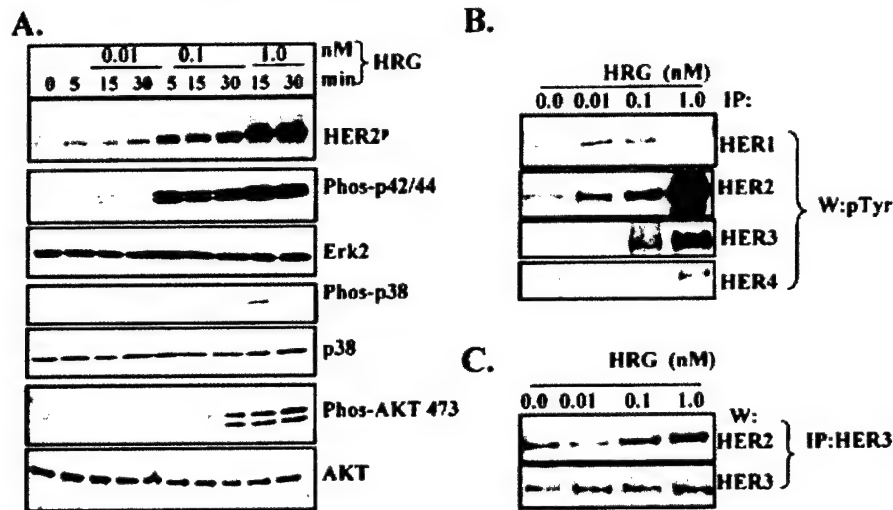


Fig. 4. HRG has a dose-dependent effect on the activation of signaling pathways and interactions among HER members. MCF-7 cells were serum starved for 24 h and treated with or without HRG for indicated times, and activation of signaling pathways was analyzed by blotting with phosphospecific antibodies. **A:** Cell lysates were blotted with anti-phosphotyrosine mAb; anti-phospho-p38^{MAPK}; anti-phospho

p42/44^{MAPK}, or anti-phospho Akt, and subsequently reprobed with anti-p38, anti-ERK, and anti-Akt antibodies. **B:** MCF-7 cell lysates (2 mg protein) were immunoprecipitated with antibodies against HER1, HER2, HER3, and HER4 and blotted with anti-phosphotyrosine antibody. **C:** HRG-treated lysates were immunoprecipitated with HER3 and blotted with antibodies against HER2 and HER3.

SHP-2 (Manes et al., 1999). Both, vector-control and SHP-2 C/S expressing MCF-7 cells were treated with 0.01 nM or 1 nM HRG for 30 min, and cell lysates were immunoblotted with phospho-specific antibodies

against FAK Tyr-577 and paxillin Tyr-31 (Fig. 7A). In vector-transfected cells, 1 nM HRG decreased the phosphorylation of FAK Tyr-577 and paxillin Tyr-31. There were no changes in the tyrosine phosphorylation

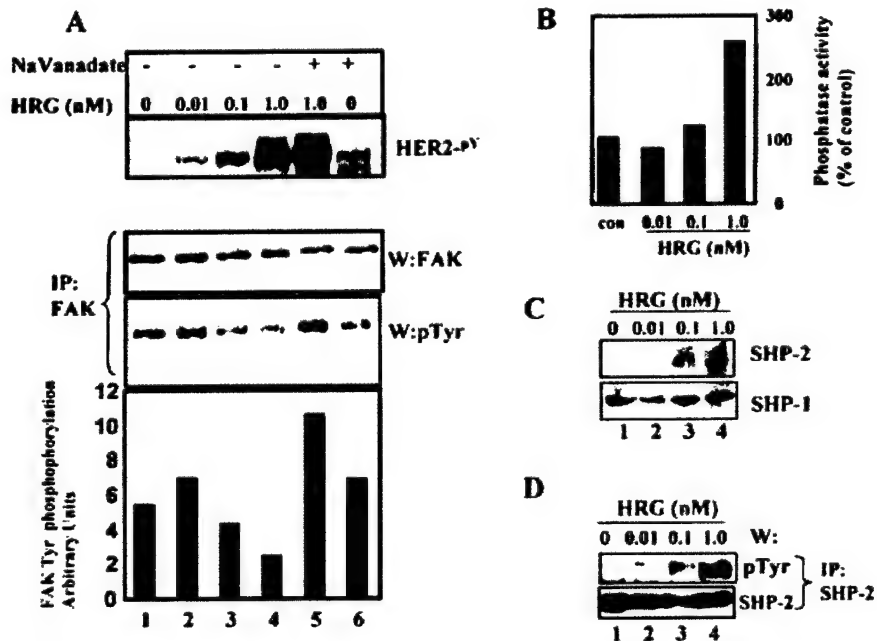


Fig. 5. HRG stimulates tyrosine phosphatase activity in a dose-dependent manner. **A:** MCF-7 cells were treated with various doses of HRG for 30 min. Some cells were pretreated with 0.5 mM sodium vanadate for 15 min, followed by 30 min of HRG treatment. HER2 and FAK were immunoprecipitated and blotted with anti-phosphotyrosine antibody. **B:** Total lysates from HRG-treated cells was analyzed for phosphatase activity using a phosphatase assay kit. Phosphatase

activity was expressed as the percentage of activity in the control untreated cells. **C:** Cells were labeled with ³²P-orthophosphate, SHP-1 and -2 were immunoprecipitated, and the status of their phosphorylation was analyzed by autoradiography. **D:** MCF-7 cells were treated with various doses of HRG, and SHP-2 was immunoprecipitated and analyzed by blotting with anti-phosphotyrosine antibody. Blot was stripped and reprobed with SHP-2 antibody as a loading control.

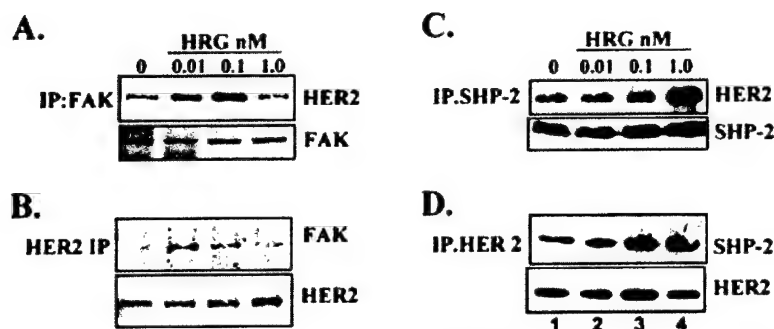


Fig. 6. HRG initiates formation of distinct signaling complexes containing HER2, FAK, and SHP-2 in a dose dependent manner. MCF-7 cells were serum-starved for 24 h and treated with 0.01, 0.1, or 1 nM HRG for 30 min. **A:** Cell lysates were immunoprecipitated with anti-FAK antibody, followed by blotting with antibodies against HER2 or FAK. **B:** Cell lysates were immunoprecipitated with anti-HER2 antibody, followed by blotting with antibodies against FAK and HER2. **C:** Cell lysates were immunoprecipitated with anti-SHP-2 antibody,

followed by blotting with antibodies against HER2 and SHP-2. **D:** Cell lysates were immunoprecipitated with anti-HER2 antibody, followed by blotting with antibodies against SHP-2 and HER2. Bottom panels of each figure represent Western analysis using the same antibodies used in immunoprecipitations, which also serve as internal loading controls. Results shown are representative of three independent experiments.

of these residues in SHP-2 mutant cells, implying a role for SHP-2 in the dephosphorylation of these residues (Fig. 7A). The lack of dephosphorylation of FAK in the SHP-2 C/S expressing MCF-7 cells was not due to defect in HRG signaling since HER2 was phosphorylated in a similar fashion as control cells (Fig. 7A, upper panel).

These observations suggested that a high dose of HRG can induce a motile phenotype, possibly by dissolving the mature and more stable focal adhesion contacts through dephosphorylation of FAK and paxillin via SHP-2. To test this hypothesis *in vivo*, we next analyzed FAK Tyr-577 and paxillin Tyr-31 tyrosine phosphorylation in SHP-2 C/S mutant cells treated with or without HRG. As shown in Figure 7B, SHP-2 C/S expressing MCF-7 cells exhibited more focal points and FAK Tyr-577 and paxillin Tyr-31 was predominantly localized to the focal points at all the concentrations of HRG. Unlike in MCF-7 cells where 1 nM HRG dramatically reduced the staining of FAK Tyr-577 and paxillin Tyr-31 (Fig. 3A,C), HRG failed to dephosphorylate FAK Tyr-577 and paxillin Tyr-31 in SHP-2 C/S expressing MCF-7 cells. Interestingly, 1 nM HRG resulted in more accumulation of focal points at in SHP-2 C/S expressing MCF-7 cells. These results suggest that a fully functional SHP-2 was needed to dissolve the well-formed focal contacts and to form new ones in response to 1 nM HRG.

DISCUSSION

Accumulating evidence suggests that the HRG pathway is involved in the progression of breast cancer cells to a more invasive phenotype and that this may involve reorganization of cytoskeleton architecture (Sepp-Lorenzino et al., 1996; Tang et al., 1996; Adam et al., 1998). Here we investigated the effects of HRG-induced early signaling on the focal adhesion proteins FAK and paxillin. Our findings suggest that HRG differentially regulates the tyrosine phosphorylation of focal adhesion proteins in a dose-dependent manner, but not all tyrosine sites are targets of HRG signaling. HRG has no effect on the FAK autophosphorylation site Tyr-397. However, a high dose of HRG increased migratory

potential of MCF-7 cells and induced dephosphorylation of FAK at Tyr-577 and -925, while suboptimal doses of HRG induced phosphorylation of FAK Tyr-577 and induced a well-defined focal point in breast cancer cells. These results suggest that extracellular HRG, even at a very low dose, affect cytoskeleton signaling, leading to distinct phenotypic changes with a role in adhesion. In contrast, 1 nM HRG activates a distinct set of signaling molecules with a potential role in migration. *In a very recent study* Lu et al. (2001) reported that growth factor, EGF dephosphorylate FAK, downregulate FAK kinase activity and such changes in FAK phosphorylation are essential for EGF induced invasion and motility. The results from the current study that HRG dephosphorylate FAK taken together with the EGF study results (Lu et al., 2001) strongly suggests that EGF family growth factor early signal transduction events involve dephosphorylation of FAK and such event plays an important role in the tumor cell invasion and motility.

Interestingly we observed HRG stimulation of tyrosine phosphatase activity in a dose-dependent manner. Activated phosphatase(s) may contribute toward the observed HRG-mediated dephosphorylation of FAK tyrosine residues. Experiments with the tyrosine phosphatase inhibitor sodium vanadate support the involvement of Tyrosine phosphatases in HRG-induced cytoskeleton signaling. The phosphatases SHP-1 and -2 were earlier shown to associate with HER receptors (Vogel et al., 1993; Tomic et al., 1995). However, in MCF-7 cells, 1 nM HRG primarily activated SHP-2. Similarly, 1 nM HRG but not 0.01 nM HRG triggered tyrosine phosphorylation of SHP-2 and its association with HER2. FAK activity was also implicated in turnover of focal points, and its disruption increased stability of the focal points (Ilic et al., 1995). Insulin and insulin-like growth factor-1 reduce tyrosine phosphorylation of FAK and paxillin in several cell types (Ouwens et al., 1996; Guvakova and Surmacz, 1999) and SHP-2 also regulates FAK activity in cells stimulated by insulin and insulin-like growth factor-1 (Yamauchi et al., 1992; Vial et al., 2000). Since higher concentrations of HRG caused a motile phenotype with formation of small focal points and decreased phosphorylated FAK staining, such

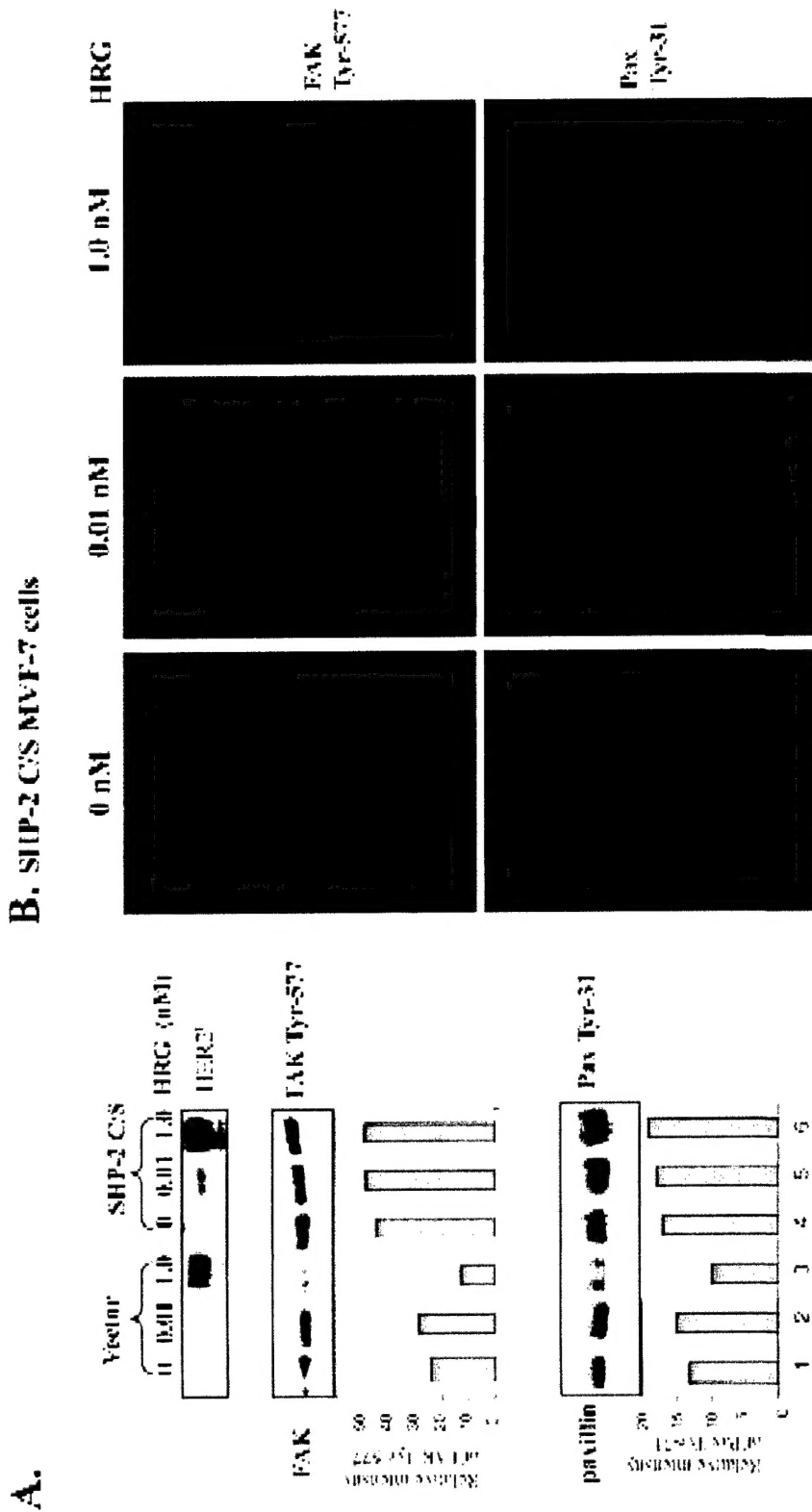


Fig. 7. Dominant-negative SHP-2 blocks HRG-mediated dephosphorylation of FAK and paxillin. **A:** MCF-7 cells expressing vector (lanes 1-3) or SHP-2 C/S (clone #14) (lanes 4-6) were serum-starved, and treated with HRG for 30 min, and cell lysates were immunoprecipitated with anti-FAK and anti-paxillin antibodies. Tyrosine phosphorylation was analyzed by Western blotting with anti-FAK Tyr-577 or anti-paxillin Tyr-31 antibody. **B:** Dominant-negative mutant (SHP-2 C/S clone #14) blocks 1 nM HRG-mediated changes in focal adhesions. MCF-7 cells stably expressing SHP-2 C/S (32) were serum starved and treated with 0, 0.01, 1.0 nM HRG for 30 min. Cells were co-stained with antibodies against FAK Tyr-577 (red color, upper panel) or paxillin Tyr-31 (red color, lower panel) and vinculin (green). Vinculin was used as a marker for focal adhesions. Cells were analyzed by confocal microscopy. Only localization of FAK Tyr-577 or paxillin Tyr-31 was shown in the figure. Note accumulation of well-formed focal contacts in SHP-2 clones even after 1 nM HRG treatment.

regulatory events may also promote cell motility. As HRG is secreted from stromal cells in mammary epithelial cells, the observed dose-dependent regulation of cytoskeleton signaling in epithelial cells may have a natural role in mammary gland development and ductal formation. It is tempting to speculate that a gradient of HRG molecules between stromal and epithelial cells also elicits distinct cytoskeleton signaling within the clusters of epithelial cells.

Tyrosine phosphorylation and dephosphorylation of paxillin were also altered by growth factor stimulation and cell adhesion and also during Src-mediated transformation (Turner, 1998). At a high dose HRG promoted dephosphorylation of paxillin at Tyr-31 and affected its localization from focal points; at a lower dose, HRG increased the phosphorylation at Tyr-31, which was predominately localized to focal adhesions. Recently, it was shown that increased tyrosine phosphorylation of paxillin- α reduces haptotactic cell migration and transcellular invasive activities in several experimental systems (Yano et al., 2000). We have previously shown that 1 nM HRG enhances serine phosphorylation of paxillin (Vadlamudi et al., 1999b), upregulates paxillin expression (Vadlamudi et al., 1999a), and increases the migratory potential of breast cancer cells (Adam et al., 1998). The results from the present study also indicate that a selective reduction in the phosphorylation of paxillin at Tyr-31 plays a role in HRG-mediated stimulation of cell motility. Potentially, regulation of paxillin tyrosine phosphorylation may have a role in the dissolution of focal points or redistributing signaling complexes. These events could be further affected by the spatial organization of different molecules in the focal adhesion complexes and the molar ratios of available ligand molecules and HER.

The results from this study also suggest that HRG regulate FAK phosphorylation by forming distinct HER complexes depending on HRG concentration. Growth factor-induced dimerization and ensuing receptor trans-autophosphorylation results in dissociation of primary HER dimer, and subsequent formation and activation of secondary HER dimers (Gamett et al., 1997). Hence, even though HRG binds HER3 and HER4, HER1 tyrosine phosphorylation at low doses of HRG may be due to secondary dimerization of HER members. We detected no HER1 tyrosine phosphorylation at a high dose of HRG. Our results also suggest that extracellular doses of ligand affect the transphosphorylation of HERs, as HRG only induced tyrosine phosphorylation of HER1 only at a suboptimum dose (0.1 nM). In contrast, we observed predominant interaction of HER2 and HER3 at a high dose of HRG. A role for HER dimers in FAK signaling was also supported by the finding that FAK associated with HER2 in response to a low but not a high dose of HRG. This suggests that HER2-HER3 dimers play a role in increasing migratory potential via HRG, in addition to their established role in mitogenesis.

In summary, our results suggest that HRG differentially regulate signaling from focal adhesion complexes through selective phosphorylation or dephosphorylation or through association of participating components and that these regulatory events have distinct roles in stromal-epithelial communication at a molecular level.

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DIFFERENTIAL REGULATION OF FOCAL ADHESION COMPONENTS BY HEREGULIN AND HER2 PATHWAY

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Heregulin (HRG) and HER2/neu signaling pathways have been implicated in the progression of breast tumors to a more motile phenotype. Cell motility/adhesion is also controlled by Focal adhesion kinase (FAK) a non receptor tyrosine kinase which is also over expressed in breast tumors. To explore the molecular participation of FAK signaling in HRG and HER2-mediated tumorigenesis in breast cancer cells, we characterized the pattern of activation of FAK, Src and paxillin all components of functional focal adhesion complex using phosphospecific antibodies. We demonstrate that in MCF-7 breast cancer cells, HRG differentially regulate tyrosine phosphorylation of FAK, paxillin and c-Src in a dose dependent manner. At low dose, HRG induced phosphorylation FAK at Tyr -577, -925, -405 and paxillin at Tyr-31, while at higher dose HRG induced selective dephosphorylation of these sites. Interestingly HRG at higher dose induced phosphorylation of FAK at Tyr -861. HRG induced phosphorylation Src at Tyr-215 in a dose dependent manner. Confocal microscopy analysis revealed that HRG at low dose stabilized focal points, while at higher dose showed changes in cell shape, formation of new focal points. HRG induced Tyrosine phosphorylation and association of Tyr phosphatase PTP1D with HER2 at higher dose. Analysis of active HER2 expressing NIH3T3 cells showed increase in the phosphorylation of FAK at Tyr-861 and Src at Tyr-215 compared to the control NIH3T3 cells. HER2-over-expressing breast tumors also showed elevated phosphorylation of FAK Tyr-861. We conclude that HER2, HRG system differentially regulate signaling from FAK by selectively dephosphorylating or activating some tyrosine residues and thus increase their migratory potential rather than adhesion. Selective upregulation of Fak Tyr-861 and Src-215 suggest that HRG/HER2 system utilize novel, yet to be identified pathways. Phosphospecific antibodies against the above signaling molecules may potentially be used as an effective reagents to screen/identifying the putative metastatic/motile potential of breast tumors.